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Effects of Bifunctional Netropsin-related Minor Groove-binding Ligands on Mammalian Type I DNA Topoisomerase

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ABSTRACT. We investigated the effects of compounds with two covalently linked netropsin moieties (bis-netropsin) on the function of mammalian type I DNA topoisomerase (topo I) *in vitro*. We initiated these studies because earlier studies had shown that certain bis-netropsins possess a several-fold higher antitumor and antiviral activity than netropsin. We confirmed that the parent compound netropsin, but not its bifunctional derivatives, induce supercoils in closed DNA. We determined that bis-netropsins inhibit the binding of topo I to DNA more efficiently than netropsin and that bis-netropsins but not netropsin induce specific DNA strand cleavage in the presence of topo I. We discuss a model explaining the different effects of netropsin and bis-netropsins on topo I. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:309–316, 1997.

KEY WORDS. netropsin; bis-netropsin; chromomycin A3; mammalian DNA topoisomerase I; cleavable complex

Mammalian topo I,¶ an enzyme composed of 765 amino acids, is involved in all genetic reactions requiring an alteration of the topological state of DNA such as replication, transcription and recombination (reviewed in [1]).

Topo I performs a reaction cycle of four distinct steps: (i) binding to double-stranded DNA, with a weak preference for certain nucleotide sequences [2]; (ii) cleavage of one of the two DNA strands by forming a covalent protein–DNA intermediate between a specific tyrosyl residue and the 3′-phosphate at the break site; (iii) the unbroken strand is then thought to either pass through the enzyme-operated nick [3] or to rotate freely [4] to relax both overwound and underwound DNA duplexes; and (iv) the covalent protein–DNA linkage is finally opened, accompanied by the resealing of the phosphodiester backbone. No external energy is required for the breakage and rejoining steps as the energy of the phosphodiester bond is conserved.

Several antitumor and antiviral drugs interfere with the topo I reaction cycle. The best known of these agents is camptothecin, which specifically affects the rejoining step of the topo I reaction and therefore stabilizes the covalent protein–DNA intermediate known as the cleavable complex. Camptothecin selectively kills cells in the S phase of the cell cycle because topo I performs a major function during DNA replication [5]. A second class of compounds affecting the function of topo I includes DNA-binding compounds such as distamycin A, the Hoechst dyes 33258 and 33342 and netropsin. These agents bind to the minor groove of DNA with a specificity for DNA regions including 4–5 central base pairs with adenine and thymine nucleotides. An additional well-studied minor groove-binding drug is chromomycin A_3, which preferentially recognizes DNA sites with central GGCC nucleotides. Several minor groove-binding drugs possess broad antiviral and antitumor activities [6, 7].

The molecular basis of topo I inhibition by some minor groove-binding drugs has been extensively investigated. For example, distamycin A inhibits the function of topo I mainly by blocking DNA sites and preventing the binding of topo I to recognition sequences [8, 9]. Conversely Hoechst dyes at intermediate concentrations function much like camptothecin by interrupting the breakage-reunion cycle by stabilizing the reversible cleavable complex. However, the sites of cleavage induced by Hoechst dyes are different from those induced by camptothecin and are characterized by a high content of adenine and thymine nucleotides [10]. In contrast, netropsin does not or only very weakly induces cleavable complex formation, even though it interacts with DNA sequences similar to those recognized by the Hoechst dyes [10]. Lown *et al.* [11] synthesized a number of netropsin analogues in which two

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¶ *Abbreviations*: Topo I, type I DNA topoisomerase; bis-nt, bis-netropsin; MAR, matrix attachment region; PCR, polymerase chain reaction; SDS, sodium dodecylsulfate; DMSO, dimethyl sulfoxide.

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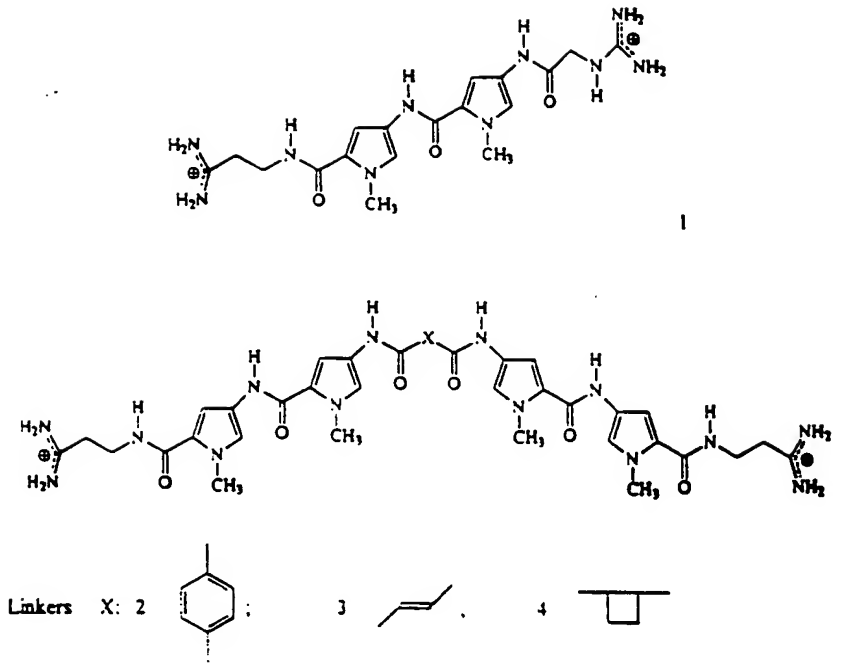

FIG. 1. Chemical structures of netropsin (1) and of bifunctional drugs related to netropsin containing the linkers (2) *p*-phenylene (Bis-Nt-2-269), (3) a trans-olefinic group (Bis-Nt-3-273) and (4) trans 1,2 cyclobutane (Bis-Nt-3-27).

netropsin units are linked with chemical groups of a different nature. Because each netropsin unit may bind to DNA, the dimeric derivatives (bis-nts) are, in principle, capable of bidentate interaction with DNA. Some of the bis-nt analogues have antitumor and antiviral activities that are significantly higher than those of the original compound [11, 12]. One possible reason for this difference is that the introduction of certain chemical groups linking the two netropsin moieties may increase the lipophilicity and membrane permeability of some of these compounds. In addition, the joining of two netropsin moieties could enhance the specificity of the ligand–DNA interaction. In fact, several of these bis-nt compounds possess a selectivity for the minor groove of AT stretches in DNA and bind to DNA with an affinity similar to that of the natural product ($K_a \sim 10^7\ M^{-1}$) [13–15].

Interestingly, previous studies on the topology of closed circular DNA have revealed a major difference in the mode of DNA binding between netropsin and its dimeric derivatives. DNA-bound netropsin causes significant changes in the linking number of DNA with concomitant changes in the superhelicity of DNA. It has been estimated that a bound netropsin molecule increases the average helical twist by 9.5°–10.5°, thus inducing supercoiling in closed DNA molecules [16]. One would expect that bifunctional netropsin analogues may cause even larger effects in DNA supercoiling. Surprisingly, however, these analogues show smaller effects in DNA overwinding [17] or fail to induce positive supercoils [16]. This finding suggests that the mode of interaction with DNA might be different with bifunctional and monofunctional netropsins.

These findings prompted us to compare the effects of netropsin and its dimeric derivatives on the function of topo I. For our investigations, we selected three novel bis-nt compounds (Fig. 1) to study their effects on topo I activity *in vitro*. We selected these compounds because previc studies had shown that they possess several-fold higher a titumor and antiviral activity than does natural netrops [11]. In particular, compounds Bis-Nt-2-269 and Bis-Nt-273 (Fig. 1) have the highest activity against herpes vir and vaccina virus among a larger series of bifunctional n tropsin derivatives [12], and these pharmalogical effec may be due to influences on topo I activity. As a contr we included chromomycin A_3 in our experiments becau this minor groove-binding agent has different DNA bin ing specificities and preferentially binds to DNA sites wi central GC base pairs [18].

MATERIALS AND METHODS

DNA

The plasmid DNA used in this study was pMII (ca. 5.8 kl a pUC18 derivative carrying the 2.9-kb Bam HI–Eco fragment MII from the MAR of the human TOP1 ge [19]. Fragment MII is comprised of approximately 68% a enine and thymine nucleotides. Plasmid pMII was purifi from bacteria by CsCl gradient centrifugation according standard procedures [20]. For filter-binding studies, pM was linearized by Eco RI restriction and end labeled by t fill-in reaction using the Klenow fragment of DNA po merase I in the presence of $\alpha(^{32}P)$dATP. For the DN cleavage reaction, we selected a 466-bp segment of the M region for amplification by PCR using an appropriate set oligonucleotide primers, one of which was 5′ end labe with γ-(^{32}P)-ATP by polynucleotide kinase [20].

DNA Ligands

Chromomycin A_3, netropsin and camptothecin (lactc form) were obtained from Sigma Chemie (Deisenhof

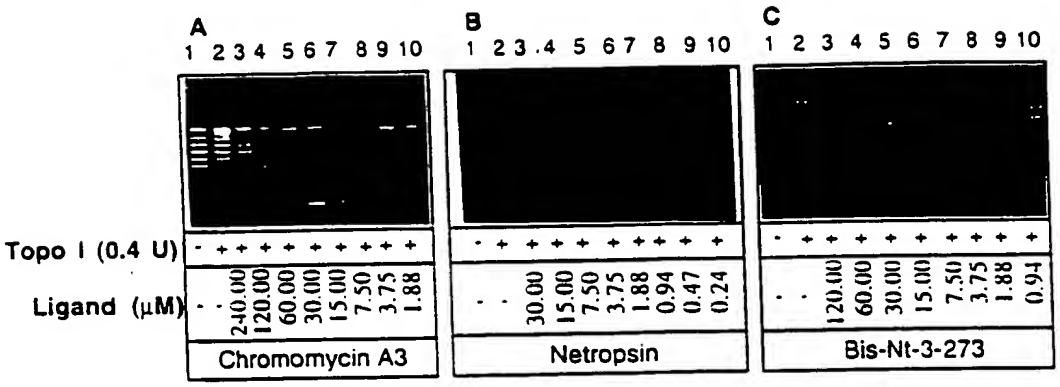

FIG. 2. Induction of supercoils into closed DNA. Relaxed plasmid pMII (lane 1 in A–C) was incubated with topo I (lane 2 in A–C) in the presence of the indicated concentrations of ligands (lanes 3–10 in A–C). DNA was extracted by phenol-chloroform, concentrated by ethanol precipitation and analyzed by agarose gel electrophoresis in the presence of 0.35 μM chloroquine. The gels were stained with ethidium bromide.

Germany) and Hoechst 33258 from Hoechst (Frankfurt, Germany). The bis-nt derivatives (see Fig. 1) were prepared and characterized as described by Lown *et al.* [11].

Enzyme Assay

Topo I was purified from calf thymus or from HeLa cells according to Strausfeld and Richter [21]. We detected no differences between the two preparations and shall therefore not distinguish between the calf and the human enzyme in the Results section. One enzyme unit is defined as the activity fully relaxing 500 ng pMII for 30 min at 37°C. Standard topo I reactions were performed in 50 μL volumes with 5% glycerol, 20 mM Tris-HCl (pH 7.8), 0.5 mM dithioerythritol, 100 mM NaCl, 5 mM $MgCl_2$ and 0.05 mg/mL bovine serum albumin using the amounts of DNA and enzyme specified below. Reactions were stopped by addition of an equal volume of 0.5% SDS in 10 mM Tris-HCl, 1 mM EDTA (pH 7.8). DNA was prepared by phenol-chloroform extraction and concentrated by ethanol precipitation.

The topology of DNA was investigated by gel electrophoresis using 1% agarose in TAE buffer (40 mM Tris-acetate, 5 mM sodium acetate, 2 mM EDTA; pH 8) for 12–16 hr at 1.5–2 V/cm. The gels were stained by ethidium bromide.

Filter Binding Assay

Linearized, (^{32}P)-3′-end-labeled pMII-DNA was first kept on ice for 5 min with or without DNA binding agents and then incubated at 37°C for 30 min with 20 units of topo I. Reaction mixtures were passed through nitrocellulose filters (BA 85; Schleicher and Schuell) as described by Gruss *et al.* [22]. The filters were dried and counted in a liquid scintillation counter.

Cleavage Reaction

PCR-amplified MII–DNA fragments, labeled by (^{32}P)-phosphate at one 5′ end, were incubated with 1 unit topo I under standard conditions in the presence of 100 μM camptothecin in DMSO (0.1% final solution) and the indicated amounts of minor groove-binding drugs (see below) as detailed by Richter and Ruff [23] who reported that DMSO in these concentrations used did not affect the topo I reaction. After 30 min at 37°C, the reaction was interrupted by 0.5% SDS followed by a treatment with 20 μg/mL proteinase K at 55°C. DNA was extracted by phenol-chloroform, concentrated by ethanol precipitation and resuspended in 60% formamide buffer. The denatured DNA was investigated on a 4% polyacrylamide/urea gel and analyzed by autoradiography. Sequencing reactions were run in parallel.

RESULTS

Induction of Superhelical Turns

As mentioned in the introduction, netropsin induces changes in superhelical turns in closed circular DNA [16], whereas certain bifunctional netropsin derivatives cause weaker effects or fail to change the topology of DNA [14, 17]. To determine whether the three compounds selected for the present study (Fig. 1) affect the topology of closed DNA, we used partially relaxed pMII (500 ng) as a substrate for the binding of netropsin or its dimeric derivatives. Topo I was included in the drug-binding reaction mixture for the detection of topological changes. Thus, drug-induced positive supercoils were released after the DNA had been purified from drugs and enzyme.

We found by gel electrophoretic analyses that changes in the superhelical density of the pMII substrate can already be detected at a concentration of 0.24 μM netropsin (compare lanes 2 and 10 in Fig. 2B), corresponding to a calculated ratio of approximately 1 molecule ligand/60 bp of DNA. The superhelical density further increases with higher drug concentrations, reaching a maximum at 7.5 μM netropsin (lane 5, Fig. 2B). Under similar conditions the netropsin derivative Bis-Nt-3-273 has little effect on the superhelical density of the pMII substrate at concentrations of 7.5–15 μM. For unknown reasons, the effects of bis-nts on DNA topology are best observed at intermediate drug concentrations (Fig. 2C). Identical data were obtained with the other two bifunctional netropsin derivatives in

vestigated (not shown). We could thus confirm previous studies with other bis-nt derivatives [14] and demonstrate a clear difference in the mode of DNA binding between netropsin and its dimeric analogs.

In Fig. 2, we have included the results of an experiment with chromomycin A_3 and show that this compound also induces topological changes in closed DNA but only at concentrations exceeding 7.5 μM (Fig. 2A).

Inhibition of Topo I Activity

To determine the effect of minor groove-binding drugs on topo I activity, we added enzyme (1 unit) to reaction mixtures containing 500 ng pMII–DNA and increasing amounts of the drugs investigated. The DNA was extracted after incubation times of 30 min and processed for analysis by agarose gel electrophoresis.

Plasmid pMII remained highly supercoiled when treated with topo I in the presence of approximately 7.5 μM chromomycin A_3 or netropsin (Fig. 3A, B). This result may at least be partly due to the induction of supercoils by bound drugs. However, a comparison with the data in Fig. 2 also suggests an effect of these two drugs on the enzyme function because superhelical DNA appeared in the enzyme inhibition assay at somewhat lower drug concentrations than required for the induction of supercoils.

The experimental situation was much clearer when bis-nt derivatives were used because these drugs induced weaker or no supercoiling (Fig. 2). Therefore, inhibiting effects on enzyme activity could be directly deduced from the gel electrophoretic data. The experiments showed th agents Bis-Nt-2-269 and Bis-Nt-3-273 completely inhibit topo I activity at concentrations of 3.75–7.5 μM (Fig. 3 D), whereas Bis-Nt-3-27 was inhibitory only at mu higher drug concentrations (Fig. 3E). A drug concentrati of 3 μM corresponds to a calculated ratio of approximat one molecule ligand per 5 bp of DNA, but we have r directly determined the fraction of the drugs bound DNA. Nevertheless, the high concentration required for inhibition of enzyme activity suggests that the mechani of drug action may be due to a blockade of topo I bindi sites on DNA, as has been shown for distamycin A [9].

To investigate this possibility, we performed filter bin ing assays with 3′-end-labeled linear pMII DNA (5 ng) the presence of topo I (1 unit) and increasing ligand co centrations. Free DNA passes through nitrocellulose filte whereas protein–DNA complexes are trapped on the fil and can be quantified by a determination of retained r dioactivity. We found that the amount of radioactive DN retained on filters by bound topo I (100% in Fig. 4) c creased in a concentration-dependent manner after pre cubation of DNA with the minor groove-binding dru Compounds Bis-Nt-2-269 and Bis-Nt-3-273 were most a tive in suppressing the binding of topo I, but approximat 10 times more Bis-Nt-3-27 was necessary to achieve a sim lar inhibition of enzyme-DNA interaction (Fig. 4). The results reflect the enzyme inhibition data shown in Fig. where Bis-Nt-3-27 was the weakest of the three bis-nt an logues with respect to topo I inhibition.

To suppress the binding of topo I to DNA, netropsin h

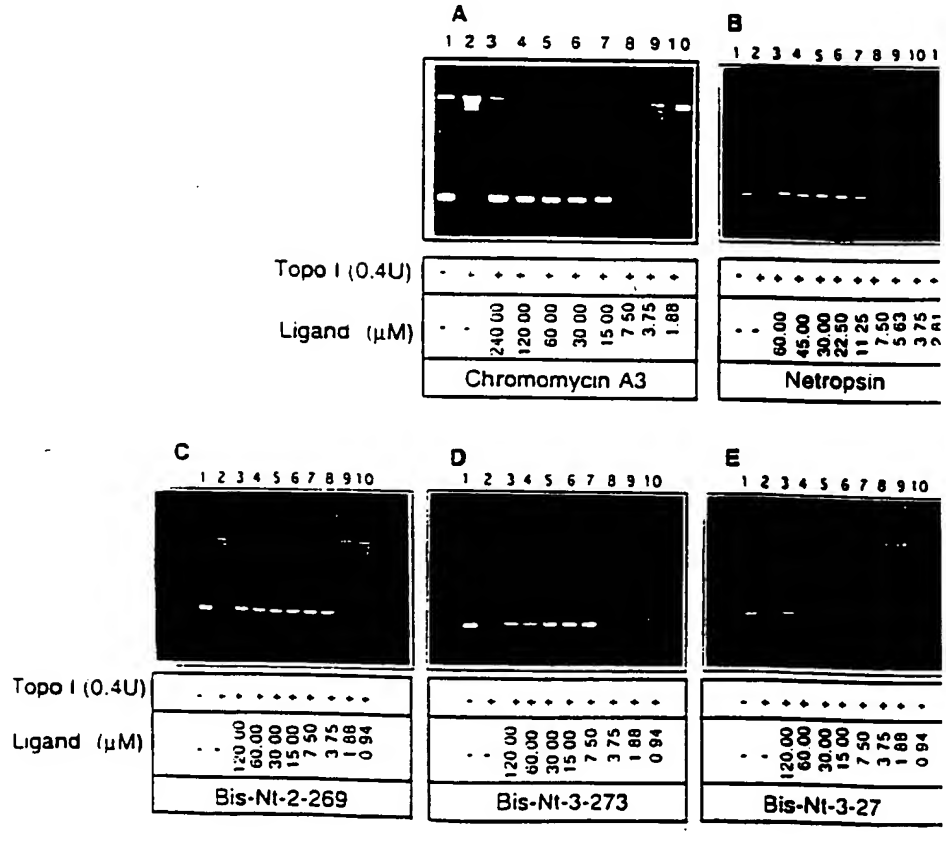

FIG. 3. Inhibition of enzyme activity. Superhelical plasmid pMII (lane 1 in A–E) was incubated under standard conditions with topo I in the absence of drugs (lane 2 in A–E) and in the presence of increasing concentrations of the indicated ligands (lanes 3–10 in A–E). DNA was extracted and processed for analysis as described in the text and the caption to Fig. 2. The experiments were performed independently at different times, resulting in slightly different distances between relaxed and supercoiled DNA molecules.

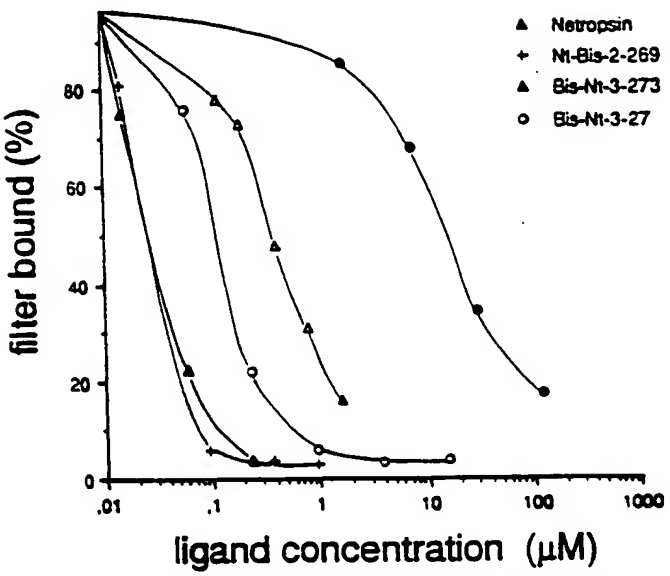

FIG. 4. Interaction of topo I with DNA. End-labeled linear pMII–DNA was incubated with topo I with and without increasing concentrations of minor groove-binding ligands. The resulting protein–DNA complexes were passed through nitrocellulose filters. The amount of radioactive DNA retained on filters in the absence of drugs was taken as 100%. Filled circles, chromomycin A_3.

to be used at significantly higher concentrations than its dimeric derivatives (Fig. 4), possibly because the dimeric derivatives of netropsin protect a larger DNA site than does the parent compound [24]. An additional result of our filter binding studies is that approximately 50 times more chromomycin A_3 than netropsin was needed to inhibit the interaction of topo I with DNA (Fig. 4). An explanation for this result may be that chromomycin A_3 prefers DNA sites with GC base pairs [6, 25], whereas topo I preferentially reacts on thymine-containing DNA regions [26].

Induction of Strand Cleavage

Chen *et al.* [10] reported that the minor groove-binding Hoechst dyes 33258 and 33342 are able to block the topo I reaction cycle by stabilizing the cleavable complex of the covalent enzyme–DNA intermediate. In spite of its similar DNA binding properties, netropsin appears to be significantly less active in this reaction. We investigated whether the bifunctional netropsin derivatives are able to stabilize the cleavable complex just as the minor groove-binding Hoechst dyes do.

To establish the experimental conditions for this reaction, we used Hoechst 33258 in a reaction with a PCR-amplified segment of the MII–DNA as a substrate and determined whether topo I induces strand breaks in this DNA sequence. The reaction mixture also included camptothecin to slow down the topo I reaction cycle (see introduction). Camptothecin induces strand breaks in an MII-related DNA sequence when tested under similar experimental conditions with topo I [23].

The result of this experiment demonstrated that intermediate concentrations of the Hoechst dye induced one DNA break in addition to those caused in the presence of camptothecin (Fig. 5A). The break induced by Hoechst 33258 was localized in a region of DNA characterized by a

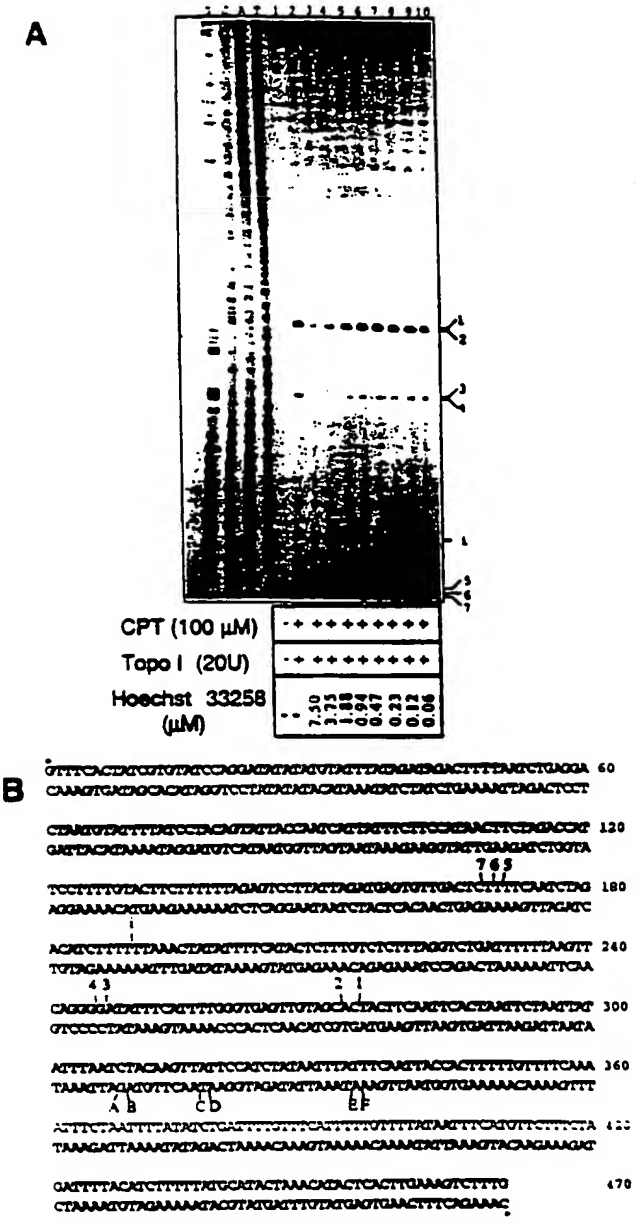

FIG. 5. Topo I induces DNA strand cleavage in the presenc of Hoechst 33258. (A) A segment of the MII–DNA se quence was labeled at the 5′ end of the upper strand an incubated without topo I (lane 1), with topo I and campto thecin (CPT; lane 2) or with topo I, camptothecin and ir creasing drug concentrations (lanes 3–10). The DNA wa extracted and analysed under denaturing conditions on sequencing gel. The products of a Maxam-Gilbert sequen ing reaction were run in parallel as markers (lanes G, C, A T). The autoradiogram of the gel is presented here. A pa allel experiment was performed using a DNA substrate ca rying the 5′ end label in the lower strand (not shown). (B Sequence of the MII–DNA segment. Strand cleavages in duced in the upper strand are labeled by arabic numbers. cut induced in the presence of Hoechst 33258. Strand clea ages induced in the lower strands are indicated by capit letters.

run of thymine nucleotides (Fig. 5B) in good agreement with the results of Chen *et al.* [10], who used a different DNA substrate. At high drug concentrations, all cleavage sites, including those promoted by camptothecin, disappeared, most likely indicating that DNA-bound Hoechst dyes prevent the association of topo I with DNA. The results shown in Fig. 5 demonstrate that the experimental system should be useful to investigate the effects of netropsin and its derivatives on the strand breakage step of the topo I reaction cycle. DNA break sites appear as two closely spaced bands on the gel, which could be due to an ambiguity in the topo I cleavage site. A second, and probably more likely, explanation is that double bands occur because of incomplete digestion of covalently bound topo I.

We first present the results obtained with increasing concentrations of chromomycin A_3 and show that relatively high concentrations of this ligand were required to inhibit the action of topo I (Fig. 6A). Significantly, all camptothecin-induced breaks disappeared when the drug concentrations exceeded the value of 3.75 μM. No strand breaks in addition to those induced by camptothecin were detectable in the presence of chromomycin A_3.

Netropsin and the netropsin derivative Bis-Nt-3-27 clearly inhibited topo I activity at lower drug concentrations (ca. 1 μM) than did chromomycin A_3 (Fig. 6B, C). Interestingly, the camptothecin breaks, labeled as band 3/4 in Fig. 6, disappeared at very low drug concentrations, whereas significant higher concentrations were required to block the DNA strand breaks at bands 1/2 and 5/7. This finding may indicate that the DNA site characterized by band 3/4 is a strong binding site for netropsin and, at the same time, a preferred topo I reaction site. Sequence comparisons (see Fig. 5B) reveal an AT-rich region next to the break, which could well serve as a preferred binding site netropsin [27, 28]. The other two break sites and flank regions may have a lower affinity for netropsin and w therefore blocked at higher ligand concentrations.

The effects of compounds Bis-Nt-2-269 and Bis-Nt 273 differed from those of the parent molecule in seve respects (Fig. 6D, E): first, inhibition of topo I occurrec very low drug concentrations (<0.47 μM); second, all DI breaks appeared to be erased at similar drug concentratic with no preference for band 3/4; third, and most interc ingly, both ligands induced an extra strand break at a quence position that was also cut in the presence Hoechst 33258 (see Fig. 5A). This extra strand break m be more efficiently induced by the Hoechst dye, but it v clearly and reproducibly detectable in the presence of gands Bis-Nt-2-269 and Bis-Nt-3-273. This result suppo the notion that these two bis-nt analogues differ signi cantly in their functional properties from the parent co: pound and from Bis-Nt-3-27.

DISCUSSION

We have investigated the effects of netropsin and thr different netropsin derivatives on the activity of topo I *vitro*. The derivatives chosen for this study contain tv netropsin units linked by different chemical groups. Bas on previous experiments, we assume that the dimeric n tropsin agents have DNA-binding properties similar to th parent compound.

Netropsin is a minor groove-binding agent with a hig affinity for DNA sites with 4 or 5 AT base pairs [6]. Th stabilizing forces between the drug and DNA are provide

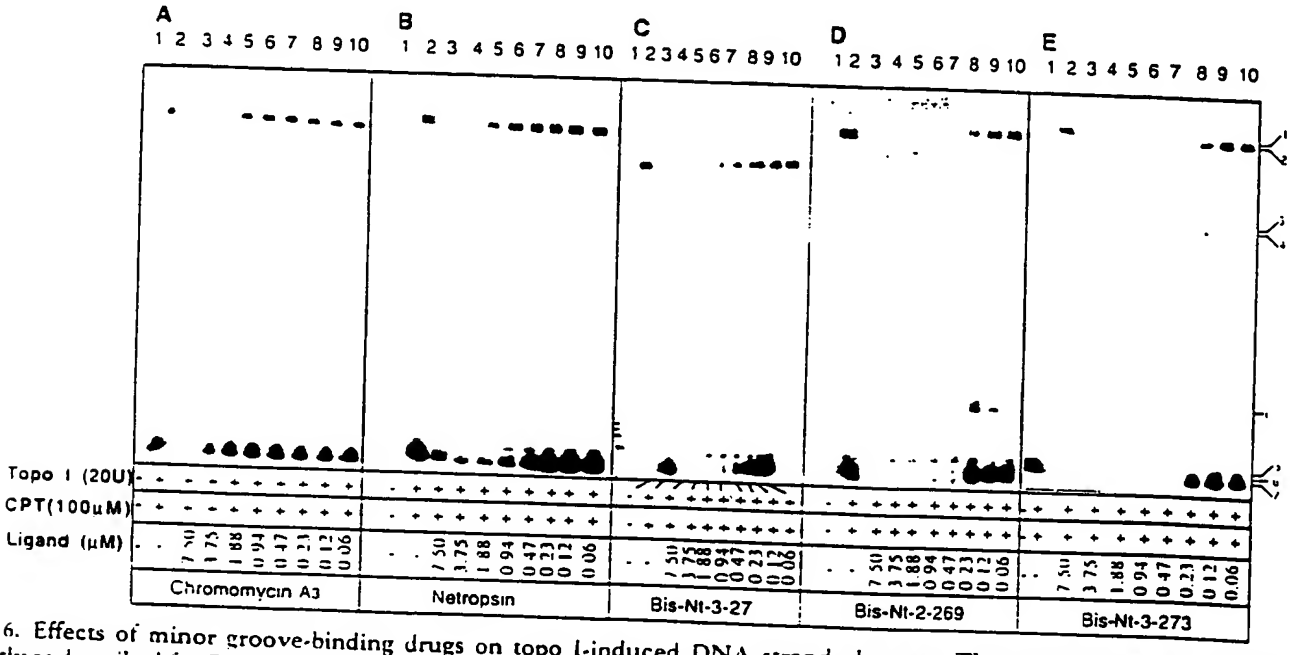

FIG. 6. Effects of minor groove-binding drugs on topo I-induced DNA strand cleavage. The experiments were performed exactly as described for Fig. 5, except that chromomycin A_3, netropsin or netropsin derivatives were used in the concentrations indicated in the figure. Lane 1, control: DNA without topo I; lane 2, DNA with topo I and camptothecin (CPT); lanes 3–10, DNA with topo I, camptothecin and ligands. Arabic numbers (right side) indicate cleavage sites in the upper strand as defined in Fig. 5B. The drug-induced cleavage site is indicated (i).

by a combination of ionic and hydrogen bonding interactions supported by van der Waals contacts [27, 28]. The bis-nt compounds probably occupy longer stretches of DNA than the monomeric parent molecule does and might even bind to recognition sequences that are interspersed with nonbinding regions. This process, however, requires that the chemical nature of the linking group provides for a spatial orientation of the two parts of the netropsin dimer for bidentate DNA binding [11].

Netropsin, unlike other minor groove-binding drugs with AT specificity, distorts the geometry of the DNA helix and locally introduces supercoils into covalently closed DNA molecules [14, 16, 17]. The reason for this is not obvious from crystallographic studies of netropsin–oligonucleotide complexes [27]. The topological changes induced by bound netropsin probably do not depend on strict sequence requirements because these changes have been observed for several natural viral DNA circles [16] and for plasmid DNA carrying unrelated sequences [14, this work]. Therefore, in solution the drug-induced topological changes probably occur at DNA sites bordering on the drug-covered DNA sections.

In any case, it is surprising that dimeric netropsin derivatives, unlike their parent compound, partially fail to induce supercoils in closed DNA [14] (Fig. 2). A possible explanation may be a slightly different mode of DNA binding of netropsin compared to bis-nts. In contrast to netropsin, which snugly fits into the minor groove of AT-rich DNA, bis-nts may have relaxed sequence requirements for high-affinity DNA binding. A less-than-optimal fit into one site may be compensated for by an additional binding to a close second site, depending on the nature of the chemical group linking the two netropsin units. Only optimal in-phase interactions between drug and DNA may induce the topological changes that lead to an increased supercoiling in closed DNA molecules. In DNA-bound bis-nts, one unit may be out of phase relative to the other unit, and the binding mode could be influenced by the structure of the chemical group between the two netropsin units. An alternative but less likely explanation is that each of the two functional parts of bis-nt has opposing effects on the geometry of the double helix, which consequently cancel each other out and therefore fail to induce supercoils.

We have stated that dimeric netropsin analogues differ in several significant ways from the parent molecules with respect to their effect on topo I. The first result was that lower amounts of compounds Bis-Nt-2-269 and Bis-Nt-3-273 than netropsin are required for an inhibition of topo I activity, which may simply be due to the fact that bifunctional ligands protect longer sections of DNA against topo I binding. This possibility is supported by filter binding assays. The data showed that bifunctional netropsin derivatives are at least ten times more efficient than the parent compound in suppressing the interaction of topo I with DNA. Bis-Nt-3-27 is less efficient in this respect than are the other two analogues, which could be due to a lower flexibility of the cyclobutane linker group (see Fig. 1), which may be less favorable for bidentate binding compared with the other analogues assayed.

The possibility that bound netropsin or netropsin analogues inhibit the access of topo I to DNA is also consistent with the structure of topo I reaction sites. DNA–bound topo I protects approximately 20 bp of DNA in footprinting experiments [2] and requires a length of 18–20 bp for strand cleavage. Experimental evidence suggests that the topo I cleavage sites are not selected at random, as shown by a comparison of a large number of topo I cleavage sites mapped on the simian virus 40 genome *in vivo* and *in vitro*. Many of the topo I cut sites are AT rich and are related to a weak consensus of 5′-A/T-A/G-A/T-T-G-3′, where the thymine nucleotide close to the 3′ end provides the phosphate group for covalent linkage to topo I [26, 29]. The preferred topo I cut sites clearly have sequence elements in common with netropsin-binding regions. In particular, if bifunctional netropsin derivatives have the relaxed binding preference suggested in this paper, drug-binding sites could well coincide with topo I cleavage sites.

We have also shown that the two most active bifunctional netropsin derivatives induce a specific topo I cut site in an AT-rich section of DNA. This site is identical with the site induced by minor groove-binding Hoechst dyes (Fig. 5) and differs from the cuts stabilized by camptothecin. Camptothecin has little effect on the specificity of topo I cutting [29], and topo I cleavage sites observed in the presence of camptothecin are usually identical with those used by topo I in the absence of camptothecin. Therefore the induction of an extra cleavage site is a consequence of the binding of bis-nt to DNA. One possibility is that bound netropsin derivatives exclude topo I from their preferred cleavage sites and force the enzyme to function at sites that are normally less favored. This explanation does not easily account for the fact that only bis-nts, but not netropsin, induce the extra cleavage site. An alternative explanation may be that, due to their more flexible and relaxed mode of DNA binding, bisfunctional netropsin analogues allow binding of topo I to neighboring DNA sites but reduce the turnover rate of the enzyme, resulting in an accumulation of cleavable complexes.

In addition to studying the AT-selective ligands, netropsin and its dimeric analogues, we have collected some data on the effects of the GC-specific dye chromomycin A_3 on topo I. We determined that much higher concentrations of chromomycin A_3 than of netropsin are required to block the binding of topo I to DNA. This determination can be most easily explained by the specificity of DNA binding because chromomycin A_3 prefers DNA sites with GC base pairs [25], whereas topo I sites are usually rich in adenine and thymine residues. Under the experimental conditions used, chromomycin A_3 blocks normal topo I cleavage sites only at very high drug:DNA ratios when a large section of DNA is covered by bound drug.

In summary, the high antiviral and antitumor activities of the bis-nt derivatives investigated [11] could at least in part be due to their effects on topo I. These effects are

twofold by blocking the binding of topo I to DNA at comparatively low drug:DNA ratios and slowing down the reaction cycle with a stabilization of the cleavable complex. The strand cleavage reaction of bis-nt compounds may be rather weak, but it would be interesting to determine whether these agents induce covalent enzyme–DNA complexes in drug-treated cells [30, 31]. A novel minor groove-binding drug, aclarubicin, which belongs to an entirely different class of chemicals, also blocks camptothecin-mediated topo I cleavage and induces drug-specific cut sites [32]. This drug action implies that the mechanism discussed above may be relevant for DNA-binding drugs other than the Hoechst dyes and bis-nts.

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